Direct Solvent–Polypeptide Interactions and their Influence on Polypeptide Conformation

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The mutarotation of poly-L-proline, poly-*O*-acetylhydroxy-L-proline and gelatin were examined in binary mixtures of anhydrous formic acid and various simple amides of differing basicity, dimethylsulfoxide and water or in ternary formic acid–water–amide mixtures. The transition involved was the inter-conversion of form I or *cis*-poly-L-proline to form II, *trans*-poly-L-proline. The I → II transition is reversible, and both forward and reverse transitions were studied. The rates, rate constants and activation energies were determined as a function of solvent composition and correlated with the intensity of interaction between solvent components. The peptide backbone–solvent interaction is comparable to the simple substituted amide–formic acid interaction. Competitive effects were reflected in marked reduction in the rate of mutarotation from that observed in inert diluents such as acetonitrile. The additional interactions due to internal peptide hydrogen bonding and special interactions of both water and hydroxyl group in the case of poly-L-hydroxyproline were readily detectable.

1. Introduction

It is well established that the immediate solvent environment of proteins and polypeptides plays a direct role in regulating their equilibrium conformations (Urnes & Doty, 1961; Singer, 1962). More specifically, many aqueous and non-aqueous mixed-solvent pairs have been used to vary the conformation of polypeptide chains from random to ordered states (Urnes & Doty, 1961), or from one ordered state to another (Steinberg *et al.*, 1960). The effect of the solvent composition can be considered to arise from either a direct inter-

action with one or the other of the solvent components or merely from alterations of the net system properties (e.g. solvent dielectric constant). Because of the many interaction possibilities and the wide range of solvent mixture properties, arising from variations in both the polypeptides and solvent mixtures, little progress has been made in understanding and systematizing the solvent–solvent and solvent–peptide interactions in terms of their effects on the final polypeptide equilibrium structures.

Our initial approach to this problem has been to consider systems in which a single type of potential polypeptide–solvent component interaction might be in direct competition with a similar limited type of solvent–solvent interaction. Chao *et al.* (1966) found that a series of non-aqueous solvent pairs based on formic acid as the proton donor and *N*-methylacetamide (NMA), *NN*-dimethylacetamide (DMA), *N*-methylformamide (NMF), *NN*-dimethylformamide (DMF), formamide, acetonitrile or dioxane as either protic bases or inert diluents, formed a set of systems in which the interaction between proton donor and acceptor decreased gradually in order of decreasing relative basicity of the carbonyl (or sulfoxyl) group on the proton acceptor. Conductivity, dielectric constant and i.r. measurements showed that the predominant interaction was that of hydrogen bonding between the hydroxy group of the acid and the carbonyl or sulfoxyl group of the base. There was no evidence for protonation of even the most basic of the amides (DMA).

Poly-L-proline (PP), poly-*O*-acetylhydroxy-L-proline (POAP) and poly-L-hydroxyproline (PHP), all exist in either of two conformations, I and II. Form I is a right-handed helix with all peptide bonds in the *cis*-configuration; form II is a left-handed helix with all bonds in the *trans*-configuration (Cowan & McGavin, 1955; Sasisekharan, 1959; Traub & Shmueli, 1963). Form I is stable in organic bases, such as pyridine, or in aliphatic alcohols, such as n-propanol, and form II is stable in water, organic acids or benzyl alcohol (Steinberg *et al.*, 1960; Katchalski *et al.*, 1963). The I $\rightarrow$ II transition involves only the *cis* $\rightleftharpoons$ *trans* isomerization about the backbone peptide bonds (Steinberg *et al.*, 1960). Isomerization is effected by interactions that reduce the partial double-bond character of the peptide bond. It was claimed that in each case hydrogen bonding and side-chain interactions were not involved in the structure stabilization in either form in the direct ways proposed for the stabilization of α-helical polypeptide structures. Thus, the I $\rightleftharpoons$ II transition in PP, POAP and PHP seemed to provide an appropriate situation in which to study the proposed competitive interaction between formic acid and amide (or dimethylsulfoxide, DMSO) in the solvent pairs and the formic acid–peptide bond interaction.

The data described below focus attention on the I $\rightarrow$ II transition in POAP, particularly as a direct example of the peptide bond–solvent com-

petitive interaction. However, two other examples of specific solvent effects, the II → I transition in gelatin, and the effect of water on the PHP I ⇌ II equilibrium in the presence of formic acid, are also discussed.

2. Details

(a) *Materials*

The polymers PP, POAP and PHP were obtained from Yeda or were synthesized directly by the method of Kurtz *et al.* (1958*a*). The PP from Yeda had a molecular weight of about 1570. The POAP and PHP were larger, with weight-average molecular weights around 15,000. An alkali precursor gelatin was used without fractionation but after desalting over mixed-bed ion-exchange resins. Each polymer was dried *in vacuo* before use.

PP and POAP were converted to form I by the addition of n-propanol to formic acid solutions. After a constant optical rotation was obtained, the polymers were precipitated from solution with ether in form I (Steinberg *et al.*, 1958).

The solvents were all prepared as described by Chao *et al.* (1966). The purity of each solvent component was checked by conductivity, dielectric-constant and refractive-index measurements. All solvent mixtures were anhydrous, except where water was purposely added.

(b) *Mutarotation*

Form I PP or POAP was dissolved directly in the appropriate solvent mixtures at a concentration of about 2 mg/ml and at the desired measurement temperature. The solutions were transferred to 1-dm water-jacketed polarimeter tubes and the optical rotation was measured at a wavelength of 365 mμ in a Rudolph Model 80 spectropolarimeter, as a function of time. Zero time was taken as the moment when the solvent was introduced into the vessel containing the polymer. Before the polarimeter tube could be filled for the first reading, it required a waiting period of 7–10 minutes before complete dissolution was effected. Since mutarotation was sometimes completed within 20–30 min, the initial rates could not be obtained with very great precision. This was particularly true for the mutarotation of PP in 100% formic acid. Most of the data reported here refer to POAP, since its mutarotation rate is slower and its solution time is a less critical factor.

The gelatin was not readily soluble in most of the solvent mixtures used. It was therefore dissolved in 100% formic acid and the second solvent component added subsequently. The specific optical rotation was measured and the final equilibrium values recorded. Final measurements were taken after 18 h.

3. Results

(a) *Mutarotation involving only backbone* cis–trans *isomerization*

(i) *The mutarotation in a non-competitive solvent*

As shown by Chao *et al.* (1966), acetonitrile interacts very weakly with formic acid and serves primarily as an inert diluent. The dielectric constant varies only from ~ 37 in pure acetonitrile to 58 in 100% formic acid, since acetonitrile has a substantial dipole moment. The specific conductances of the various formic acid–acetonitrile mixtures also change only to a small degree. Thus, the behavior of POAP in the formic acid–acetonitrile mixtures should represent only the concentration dependence of the formic acid–peptide interaction.

Figure 1 illustrates the course of the I → II transition for POAP as a function of formic acid concentration. Only a small change in mutarotation rate, from that noted for the polymer in 100% formic acid, is seen until the concentration of formic acid drops below 60% v/v. In every case examined, the final equilibrium value of $[\alpha]_{365}$ was the same after suitable refractive-index corrections, $(n^2+2)/3$, were made.

(ii) *Mutarotation in protic, competitive solvents*

There is a distinct maximum in the conductivity of formic acid–amide and formic acid–DMSO mixtures at 80% formic acid v/v (Chao *et al.*, 1966), and this appears to correspond to extensive formic acid–amide hydrogen-bonded aggregate formation. This set of solvent mixtures thus represents the situation

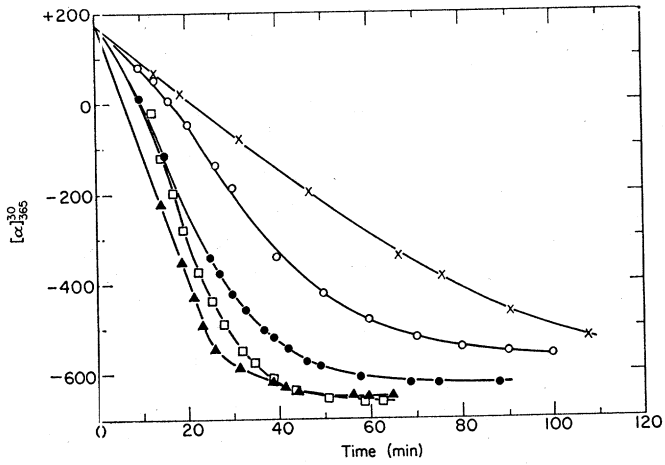


FIG. 1. Graphs showing the specific rotation of POAP as a function of time in the I → II transition in formic acid–acetonitrile mixtures: ▲, 100% formic acid (v/v); □, 90%; ●, 70%; ○, 50%; ×, 30%. All measurements at 30°, wavelength 365 mμ.

in which the amide diluent interacts with the proton donor in direct competition with the peptide carbonyl group. The POAP solutions examined were all approximately 2×10^{-2} M in peptide groups and the DMF concentrations were all greater than 1M. It was not surprising then that the DMF was found to lower the mutarotation rate very sharply. Even at a concentration of only 30% formic acid in acetonitrile, the mutarotation (Fig. 1) is markedly faster than seen in a 90% formic acid–10% DMF mixture (Fig. 2).

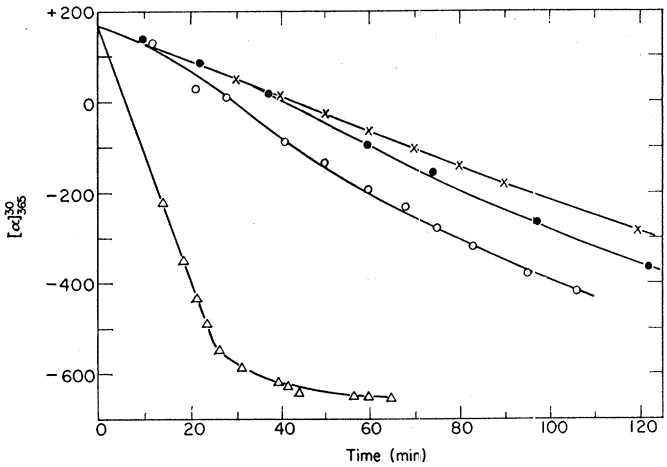


FIG. 2. Graphs showing the specific rotation of POAP as a function of time in the I → II transition in formic acid–DMF mixtures: △, 100% formic acid; ○, 90%; ●, 80%; ×, 60%. Measurements at 30°, wavelength 365 mμ.

The formic acid–DMF interaction is more intense than the formic acid–DMSO interaction, as indicated by the relative specific conductances of these mixtures and the higher basicity of the carbonyl group as compared with the sulfoxyl. A graph of $[\alpha]_{365}^{30}$ *versus* time for formic acid–DMF and formic acid–DMSO at 80% formic acid concentration (Fig. 3) demonstrates that the mutarotation rates are decreased in accordance with the increasing strength of the solvent-pair interaction.

Steinberg *et al.* (1960) proposed that the specific rotation of polyprolina or its derivatives during mutarotation could be represented as:

$$[\alpha]_t = \frac{C_{cis}}{C_0}[\alpha]_{\mathrm{I}} + \frac{C_{trans}}{C_0}[\alpha]_{\mathrm{II}}$$

where:

$$C_0 = C_{cis} + C_{trans}$$

which leads to:

$$\frac{C_{cis}}{C_0} = \frac{[\alpha]_t - [\alpha]_{\mathrm{II}}}{[\alpha]_{\mathrm{I}} - [\alpha]_{\mathrm{II}}}$$

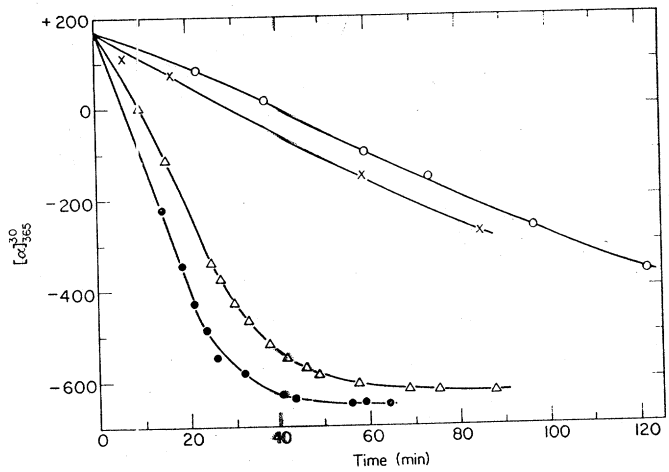


FIG. 3. Graphs showing a comparison of the I → II transition of POAP in: ●, 100% formic acid; △, 70% formic acid in acetonitrile; ×, 80% formic acid in DMSO; and ○, 80% formic acid in DMF. Measurements at 30°, wavelength 365 mμ.

First-order kinetics for the mutarotation would then require that a graph of ln $([\alpha]_t - [\alpha]_{II})$ *versus* t be linear. This was found to be so in the region from 20 to 80% conversion of form I to form II in each of the solvent systems studied. A co-operative effect in the transition was indicated when mutarotation was slowed by the addition of DMF or DMSO. The first phase (up to ~ 20% conversion to form II) was slower than the succeeding conversion, suggesting that a nucleation step was required. However, this point must be checked by other means, since it is possible that incomplete or slow dissolution of the polymer might have complicated the situation. For the purpose of comparing solvent effects, the rates and apparent first-order rate constants, were computed from the data taken in the range of 30–50% conversion to form II. Table 1 shows the apparent first-order rate constants observed at various formic acid concentrations at 30°.

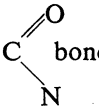
The first-order rate constants were determined at the same degree of conversion as a function of the temperature in the range from 40 to 25°C for 80% formic acid–DMF and formic acid–acetonitrile. Graphs of $\log k$ against $1/T$ were linear over the limited temperature range examined. Activation energies determined from these data are listed in Table 2. The higher values of ΔE_a are consistent with those in the literature (Steinberg *et al.*, 1958; Downie & Randall, 1959) and in agreement with the predicted value for rotation about the amide partial double bond (Pauling & Sherman, 1933). The markedly lower values seen in 100% formic acid and in formic acid–acetonitrile mixtures are not, indicating that extensive binding of formic acid

TABLE 1. First-order rate constants at 50% conversion for the form I→form II transition in poly-*O*-acetyl-L-hydroxyproline at 30°

Concentration of formic-acid			Rate constant, k, min^{-1}	
By volume %	By mole % in formic acid –acetonitrile mixture	By mole % in formic acid –DMF mixture	Acetonitrile	DMF
100	100	100	0·0855	0·0855
90	92·66	94·84	—	0·0127
80	84·86	89·10	—	0·00946
70	76·59	82·62	0·0513	—
60	67·77	75·36	—	0·00768
50	58·37	67·11	0·0302	—
30	37·54	46·64	0·0267	—

TABLE 2. Activation energies for the form I→form II transition in poly-*O*-acetyl-L-hydroxyproline in formic acid–diluent mixtures

Solvent mixture (v/v)	Apparent activation energy (50% conversion) kcal/mole
100% formic acid	9·3
80% formic acid–20% acetonitrile	16·5
90% formic acid–10% DMF	24·6
80% formic acid–20% DMF	22·4

to the polymer reduces the partial double-bond character and permits free rotation at the  bond.

In each case the final value of the rotation of POAP was the same, within experimental error, regardless of the solvent mixture or the temperature at which mutarotation was studied. A similar situation was found for PP in the same set of solvent mixtures.

(b) *Mutarotation involving hydrogen bonding in addition to* cis–trans *isomerization*

Gelatin is rich in glycine, proline and hydroxyproline and has many Pro-Hypro sequences. It forms ordered "collagen-fold" units with the same chain configuration as the poly-L-proline II structure (see Veis, 1964, or

Harrington & von Hippel, 1962, for detailed reviews). It is completely disordered in 100% formic acid (Veis & Anesey, 1959) but changes to a form with a more positive specific rotation when the formic acid is diluted with propanol (Steinberg *et al.*, 1960) or with DMF (Veis & Anesey, 1959). Veis & Anesey (1959) speculated that the change in rotation was due to the same type of *trans* → *cis* isomerization as that seen in the PP form II → form I transition. The situation is more complicated than in PP, however, because of the possibility of backbone hydrogen bonding at the residues other than proline or hydroxyproline, and the ionization of the basic side-chain residues with simultaneous suppression of the carboxyl-group ionizations. A very marked electrostatic effect led to enhanced viscosities in formic acid–DMF mixtures at formic acid concentrations above 80%. This was interpreted in terms of backbone protonation (Veis & Anesey, 1959), but in view of the lack of evidence for amide-group protonation in the formic acid–amide mixtures (Chao *et al.*, 1966) this should be reconsidered as a side-chain ionization effect. In any case, the gelatin system appeared to represent a situation in which the principal backbone effects would be those of Pro-Hypro *cis–trans* isomerization and solvent competition with backbone–backbone interactions.

As indicated earlier, solubility problems required that all solutions be prepared by dilution of gelatin solutions in 100% formic acid. The mutarotations thus observed were the reverse of the rotation changes discussed in Section 3(a).

In contrast to the PP and POAP mutarotations, the final equilibrium value of the specific rotation was solvent dependent. The equilibrium rotations are shown in Fig. 4 for the formic acid–DMF, formic acid–DMSO and formic acid–acetonitrile solvent systems at 25°C. It can be seen that in each solvent system the gelatin behaves as if there is a co-operative type of conformational transition. The formic acid concentration required for the attainment of the *trans*-disordered form is higher, the stronger the direct formic acid–diluent interaction.

(c) *A specific effect of water*

As noted earlier, the I → II transitions of POAP and PP are very rapid in 100% formic acid and proceed essentially to completion even in the presence of the amide diluents. Water acts as an analog of the amides and also slows the I → II transition. Similarly, as shown in Fig. 5, the final stable value for $[\alpha]_\lambda$ is unchanged in formic acid–water mixtures. The behavior of PHP does not follow this pattern, however, and is in distinct contrast to that of POAP and PP.

Kaufman *et al.* (1966) demonstrated that form II PHP in formic acid mutarotates rapidly to a value of $[\alpha]_D^{23}$ near $-235°$ from an initial value of

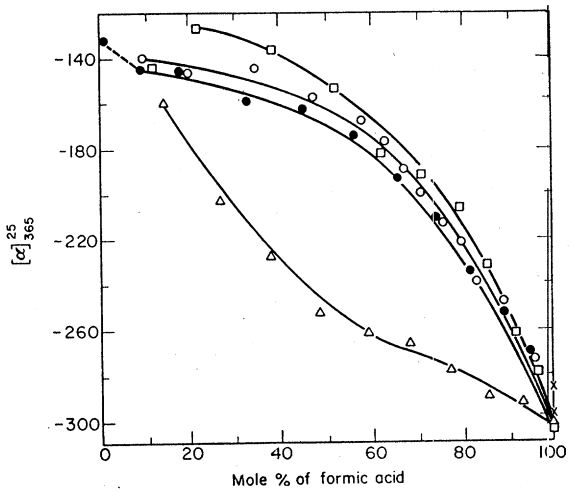


Fig. 4. Graphs showing the equilibrium specific rotations of an alkali precursor gelatin at 25° as a function of formic acid concentration (mole %) in formic acid–diluent mixtures. Diluents: △, acetonitrile; ●, DMSO; ○, DMF; □, DMA. Measurement at wavelength 365 mμ.

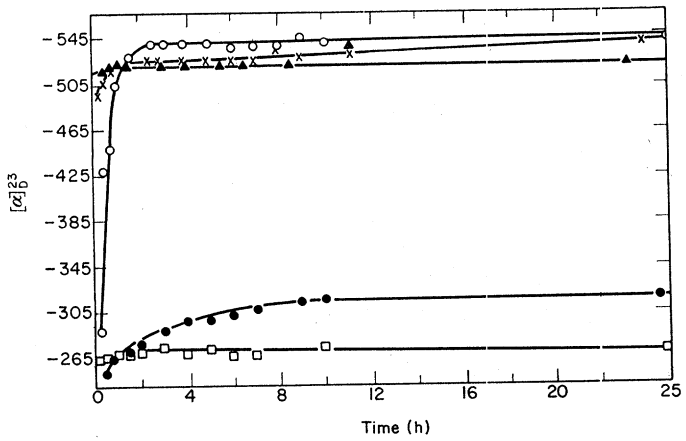


FIG. 5. Graphs showing the specific rotations of PP and PHP in formic acid–water or formic acid–DMSO systems. Transition from form I → form II. Measurements at 23° and at sodium D line (data uncorrected for refractive index changes): △, PP in 100% formic acid (mole %); ×, PP in 83% formic acid–17% water; ○, PP in 56% formic acid–44% water; ●, PHP in 56% formic acid–44% water; □, PHP in 60% formic acid–40% DMSO.

$-400°$. The addition of water to the formic acid–PHP solution shifts the equilibrium back in the direction of form II and the final stable value of $[\alpha]_D^{23}$ is a linear function of the mole fraction of water in the system (Fig. 6). These data show that the hydroxy groups of PHP must interact strongly with their neighboring peptide-bond carbonyl oxygen atoms through the direct intervention of a water molecule. Models show that in normal form II PHP, the separation of the carbonyl oxygen atom of one residue and the hydroxyl hydrogen atom of the next residue is such that a single water

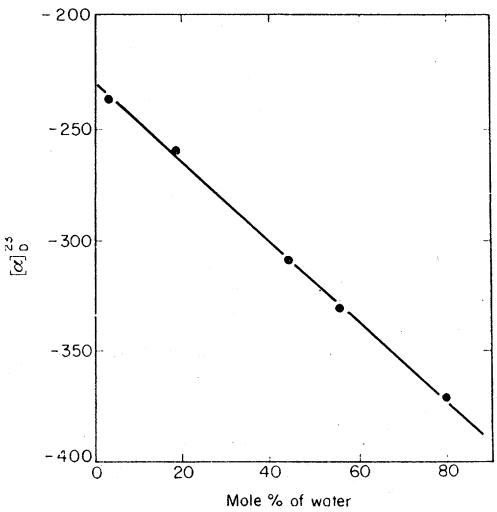


FIG. 6. Graph showing the equilibrium specific rotation, at 23° and with sodium D line, of PHP in water–formic acid mixtures as a function of the water content. (Data of Kaufman *et al.*, 1966).

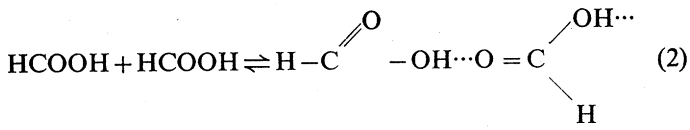
molecule can easily form a bridge consisting of two hydrogen bonds between these groups. A water bridge of this type would favor and stabilize the *trans*-configuration in PHP in aqueous systems.

The present observation that the addition of water to formic acid solutions of PHP slows the I → II mutarotation rate without altering the final value of $[\alpha]_\lambda$ is further evidence in favor of a special role for both water and the hydroxyl group in PHP *trans*-configuration stabilization.

4. Discussion

Chao *et al.* (1966) have shown that formic acid is a differentiating solvent for weak bases, such as the simple amides and dimethylsulfoxide. Anhydrous formic acid itself is an associated liquid in which auto-ionization and hydrogen-bonding reactions, (1) and (2), both take place:

$$HCOOH + HCOOH \rightleftharpoons HCOOH_2^+ + COO^- \quad (1)$$



(2)

The Hammett function, $-H_0$, for anhydrous formic acid is $-2{\cdot}19$ at 30°C (Stewart & Mathews, 1960), and the concentration of $HCOOH_2^+$ is on the order of 10^{-3} M. The addition of even a minute amount of a more basic proton acceptor shifts equilibrium (1) to the left and the protonating species concentration ($HCOOH_2^+$) drops drastically (Hammett & Deyrup, 1932; Stewart & Mathews, 1960). The i.r., volume of mixing, viscosity, dielectric constant and conductivity data (Chao *et al.*, 1966) all point to the formation of hydrogen-bond interactions between acid and proton-acceptor amide to form rather extensive amide–acid hydrogen-bonded aggregates with maximum aggregate size at about 85% formic acid v/v. Direct protonation of the amides by formic acid has not been detected at formic acid concentration of 95% or less, whereas this is easily detected in the near i.r. spectra of leveling acid solvents, such as trifluoroacetic acid or dichloroacetic acid (Hanlon *et al.*, 1963; Hanlon, 1966).

Studies on the i.r. spectra, dielectric increments and conductivities of several homo-polypeptides in anhydrous formic acid (A. Veis, unpublished results) suggest that the polypeptides, including PP and POAP, behave in a fashion entirely analogous to the simple amides. Further, different polypeptides show different intensities of interaction. A substantial amount of work remains to be done on these systems (particularly on the effect of polymer molecular weight), but it would appear that in every case one principal interaction is hydrogen bonding of formic acid to the peptide carbonyl oxygen atoms. These observations form the basic premises of the present study: (1) that conformational transitions can be brought about by direct solvent interactions and solvent "binding" at the peptide groups; and (2) that weakly proton-accepting bases act competitively to shift the binding equilibria and hence modify the conformation. The exact nature of the "binding", protonation or hydrogen bonding, does not materially change the arrangement that follows.

The examples cited in the preceding section were chosen in an attempt to delineate a series in which several potential types of competitive interaction between polypeptides and solvent-mixture components were possible and where the interactions might be distinguished from one another. These data are not, at this point, presented for the purpose of describing either the *cis–trans* isomerization at the peptide bond or the detailed nature of the polypeptide conformational transitions.

The I → II transitions of PP and POAP in formic acid represent the situation in which the only possible hydrogen-bonding interaction is at the carbonyl oxygen atom of the peptide. Dilution of formic acid with a non-competitive aprotic diluent has little effect on either the rate of the I → II transition or the final equilibrium state at the peptide bond except at relatively very high dilutions of formic acid. The rate constant in acetonitrile, as indicated in Table 1, is approximately proportional to the molar concentration of formic acid and suggests that a single equilibrium constant can describe the formic acid–peptide carbonyl oxygen hydrogen bonding interaction. The situation is drastically altered upon the addition of a protic diluent that reduces the "free" formic acid concentration, and hence shifts the formic acid–peptide interaction in favor of non-bonded peptide groups. In order for this to occur upon the addition of only a few mole per cent of amide, the peptide carbonyl in POAP or PP must be a weaker base than the simple amides. In spite of the reduced formic acid interaction and the sharply lower rate of mutarotation, the I → II transition proceeds to completion. Models show that in the extended form II *trans* configuration the C—O groups are more accessible to the solvent. Water has the same effect as the other protic diluents in formic acid.

The shift of PHP from form II to form I, a reverse mutarotation in anhydrous formic acid, appears to be strong evidence in favor of a specific interaction of the neighboring side-chain hydroxy groups with the peptide carbonyls in the anhydrous solution. Kurtz *et al.* (1958*b*) found evidence for such an interaction in the shift of the C—O absorption in the i.r. spectrum of water-free PHP to a lower frequency than that observed in POAP. This interaction is favored in the more compact *cis* form of PHP. However, at equilibrium in 100% formic acid, the PHP must be a mixture of *cis* and *trans* forms, since the specific rotation is not that expected for fully *cis* or form I PHP.

A second specific interaction is evident from the reversion of the PHP in formic acid back to form II upon the addition of water. The water diluent may have several functions. It must reduce the direct formic acid–PHP interaction in the same fashion as in PP or POAP. Similarly, water may compete in the $-OH \cdots O{=}C-$ interaction. However, in this case, the steric suitability of the $-OH \cdots H-O-H \cdots O{=}C-$ structure in form II favors this conformation.

Finally, the behavior of gelatin in anhydrous formic acid–protic diluent mixtures illustrates the effect of an additional set of interactions. Steinberg *et al.* (1960) and Veis & Anesey (1959) suggested that gelatin takes up the form I PP configuration in non-aqueous acid–diluent systems. From the behavior of POAP in formic acid–DMA, DMF or DMSO one must conclude that the frequent Pro-Hypro sequence would tend to remain in the *trans*

form in the formic acid–diluent mixtures unless, as in PHP, some specific internal interaction at these peptide linkages was favored. Since the most negative specific rotation is obtained in 100% formic acid, the likelihood of an effect such as seen in the PHP case is small. The shift in rotation to more positive values depicted in Fig. 4 probably involves the gelatin sequences that do not contain proline or hydroxyproline residues, and the structures formed depend upon internal hydrogen-bond stabilization. The more compact form I structure may maximize these interactions. The peculiar nature of this form of form I gelatin is evident in the unusual solubility properties of the product. As shown by Veis & Anesey (1959) the form I gelatin is water insoluble. In addition, we have found the product to be so stable as to resist dissolution in boiling water for several minutes, although the gelatin swells and imbibes a substantial amount of water. However, the form I gelatin is quite soluble in formic acid.

Obviously, the detailed nature of the interactions and conformational transitions described above remain to be examined in detail in each situation. It is clear, however, that the use of differentiating solvent mixtures does provide one means of separating the very complex sets of interactions into a few groups that may be distinguished from each other and examined in model systems. It may thus be possible selectively to probe differences in local polypeptide configurations based on individual peptide-bond basicities and the neighboring-group environment. Most important, water fits easily into the weak acid–protic diluent system employed.

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